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Cadmium and Proliferation in Human Uterine Leiomyoma Cells: Evidence of a Role for EGFR/MAPK Pathways but Not Classical Estrogen Receptor Pathways

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Running title: EGFR/ERK in Cd-induced uterine fibroid cell growth

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Abstract

Background: It is proposed that cadmium (Cd) is an environmental "metalloestrogen" and its action is mediated via the estrogen receptor (ER). Cd mimics the effects of estrogen in the rat uterus, and blood Cd concentrations positively correlate with ER levels in uteri of women with fibroids.

Objectives: This study explored whether Cd could stimulate proliferation of estrogen-responsive human uterine leiomyoma (ht-UtLM) and uterine smooth muscle cells (ht-UtSMCs) through classical interactions with ERα and ERβ, or by nongenomic mechanisms.

Methods: Estrogen response element (ERE) reporters, phosphorylated receptor tyrosine kinase arrays, western blot analysis, estrogen binding, and cell proliferation assays were used to evaluate the effects of Cd on ht-UtLM cells and ht-UtSMC.

Results: Cd stimulated growth of both cell types at lower concentrations and inhibited growth at higher concentrations (≥50 μM). Cd did not significantly bind to ERα or ERβ, or show transactivation in both cell types transiently transfected with ERE reporter genes. However, Cd (0.1 μM and 10 μM) activated p44/42 MAPK (ERK1/2) in both cell types, and a MAPK inhibitor, PD98059, abrogated Cd-induced cell proliferation in both cell types. Cd in ht-UtLM cells, but not in ht-UtSMC activated growth factor receptors, EGFR, HGFR, and VEGF-R1, upstream of MAPK. Additional studies in ht-UtLM cells showed that AG1478, an EGFR inhibitor, abolished Cd-induced phosphorylation of EGFR and MAPK.

Conclusions: Our results show how low concentrations of Cd stimulate cell proliferation in estrogen-responsive uterine cells by nongenomic activation of MAPK, but not through classical ER-mediated pathways.

Introduction

Cadmium (Cd), is a toxic metal and common environmental contaminant, with human exposures most commonly occurring through occupational inhalation, or by tobacco use, ingestion (food and drinking water), or inhalation of ambient air [Agency for Toxic Substances and Disease Registry (ATSDR 2008); International Agency for Research on Cancer (IARC 2012)]. Data from the National Health and Nutrition Examination Survey (NHANES 2011) show that over 60 percent of the US population have detectable blood Cd levels (range: 1.25 to 77.14 nmol/L). Chronic Cd exposure has been associated with increased lung and prostate cancers in occupationally exposed workers in the US (Verougstraete et al. 2003), and elevated levels of serum Cd correlate with human pancreatic cancer (Kriegel et al. 2006). Evidence from rodent and *in vitro* studies show a direct causal link between Cd and cancer (Jing et al. 2012; Qu et al. 2012). Molecular studies have suggested that the underlying mechanism of the carcinogenic activity of Cd is multifactorial and may include DNA damage (Zhang et al. 2010), phenotype transitioning (Benbrahim-Tallaa et al. 2009), modification of Cyp1a1 expression (Kluxen et al. 2012), Sp1 inactivation (Youn et al. 2005) and promotion of angiogenesis (Jing et al. 2012).

Recent studies have suggested that Cd is an environmental "metalloestrogen" and its effects may be mediated by the estrogen receptor (Johnson et al. 2003; Kluxen et al. 2012). The proposition of Cd being an endocrine disruptor is plausible due to reports of its wide spectrum of deleterious effects on experimental *Xenopus laevis* (Lienesch et al. 2000), mice (Ali et al. 2010), rats (Johnson et al. 2003), and the developing human reproductive tract (Kippler et al. 2012). In ovariectomized rats, Johnson et al. (2003) reported that exposure to Cd increased uterine wet weight with accompanying proliferation of the endometrium and induction of the progesterone receptor. Other investigators have found that Cd regulates progesterone synthesis in cultured

granulosa cells (Nampoothiri et al. 2007) and in pseudo-pregnant rats (Henson and Chedrese 2004). Additionally, a recent cohort study suggested a definite role for Cd in postmenopausausal breast cancer in women (Julin et al. 2012); this being consistent with prior observations of Cd's ability to transform human breast epithelial cells into a cancer phenotype *in vitro* (Benbrahim-Tallaa et al. 2009).

There is reasonable evidence suggesting that Cd may be associated with uterine disease in women (Jackson et al. 2008). Nasiadek *et al*. (Nasiadek et al. 2005) detected Cd in uterine tissue of women with leiomyoma, although the concentrations were slightly lower than surrounding myometrium. Additionally, these investigators found that tissue Cd levels correlated with levels of ER expression in leiomyoma tumors, indicating a possible link between Cd and estrogen signals (Nasiadek et al. 2011). Considering that uterine fibroids (leiomyomas, myomas) are one of the most common hormonally responsive tumors clinically affecting women of reproductive-age, it is a first-line strategy to identify potential environmental risk factors for the management of this disease (Di et al. 2008; Gao et al. 2012).

The ability to activate estrogen receptor alpha (ERα) is central to estrogen and "estrogen mimics" inducing cell proliferation in many cancers and other disease processes (Osborne and Schiff 2005). At the molecular level, estrogens, such as 17β-estradiol, bind to either ERα or estrogen receptor beta (ERβ) and function through classical or several nongenomic signaling pathways, with the latter including the pro-proliferation, mitogen-activated protein kinase (MAPK)/ERK1/2 signaling pathway (Creighton et al. 2006). The MAPK pathway is a critical regulator of cell proliferation in both normal development and tumor growth. (Dhillon et al. 2007; Osborne and Schiff 2005). Conversely, the role of ERβ has largely been associated with inhibition of proliferation or pro-apoptotic events when co-expressed with ERα; however, recent studies in

ERα-negative breast cancer cells may suggest a role of ERβ in cell survival (Leygue and Murphy 2013).

In the present study, we determined whether Cd could induce growth in estrogen-responsive human uterine fibroid and myometrial cells, and if so, did ERs mediate the effects. We first examined the effects of low and high concentrations of Cd on the growth, and then explored possible molecular mechanisms mediating any Cd-induced effects. Our results have important clinical and environmental risk implications, as well as provide evidence of a molecular mechanism of Cd-induced effects in uterine fibroid cells.

Materials and Methods

Cells and reagents

The UtLM-hTERT (ht-UtLM) cells and UtSMC-hTERT cells (ht-UtSMCs) at passage 24 were established in our lab and maintained in supplemented medium as previously described (Carney et al. 2002). Cadmium chloride ($CdCl_2$; 99.999%, #439800, Sigma-Aldrich) was dissolved in double distilled water to make a 1 M stock solution. The ER antagonist, ICI 182,780 (ICI, Sigma-Aldrich), MAPK inhibitor, PD98059 (PD, #9900, Cell Signaling) and EGFR inhibitor, Tyrphostin AG 1478 (AG, #9842, Cell Signaling) were dissolved in DMSO.

Cell proliferation assay - MTS

CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS, G3581, Promega) was used to measure cell proliferation according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates and cultured in phenol red-free DMEM/F12 medium containing 10% Charcoal/Dextran-stripped FBS for an additional 24 h, followed by various Cd treatments without or with PD (10 μM) pre-incubation (2 h).

Estrogen receptor α and β competitive binding assay

The binding affinity of E_2 and Cd to ERα and ERβ was evaluated by fluorescence polarization following the instructions provided by LanthaScreen® TR-FRET Estrogen Receptor alpha and beta Competitive Binding Assay (PV6041 and PV6042, respectively, Invitrogen). According to the manufacturer's protocol, E_2 or Cd at increasing concentrations was added to ER/Fluormone^{TM} ES2 Green mixer.

Transient transfection luciferase assays

Cells were transfected by using Lipofectamine® RNAiMAX Transfection Reagent (#13778-075, Invitrogen by Life Technology^{TM}) as reported earlier (Gao et al. 2012). Transfected cells, equipped with luciferase reporters, were maintained in medium without phenol red, containing 10% Charcoal/Dextran-stripped FBS for 24 h prior to treatment with 10 nM E_2 or Cd (0.01, 0.1, 1.0, 10, 20 μM) in the presence or absence of 1 μM ICI for 24 h. Luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Promega).

Western blot analysis

Whole cell lysates were obtained and used for western blotting as described earlier (Gao et al. 2012). The following primary antibodies for targeted molecules were diluted at 1:1000 and were purchased from Cell Signaling: Phospho-p44/42 MAPK (#9101), Phospho-44/42 MAPK Antibody (#9102), Phospho-EGF Receptor (#3777) and EGF Receptor (#2232). ECL HRP-Linked Rabbit IgG (1:5000, NA934, GE Healthcare) was used as a secondary antibody. HPRT (Hypoxanthine-guanine phosphoribosyltransferase) antibody (sc-376559, Santa Cruz) was used as a loading control. The density of the respective bands was quantitated by using a densitometer with AlphaView Software for FluorChem Systems (ProteinSimple^{TM}).

Phosphorylation of receptor tyrosine kinases (RTKs) array

In an attempt to assess the phosphorylation status of human receptor tyrosine kinases (RTKs), Proteome Profiler™ Human Phospho-RTK Array Kits (#ARY001, R&D Systems) were utilized according to the manufacturer's protocol. Briefly, cell lysates were aliquoted and incubated with the RTK array membranes spotted with 42 anti-phospho-RTK antibodies. A pan anti-phospho-tyrosine antibody conjugated to horseradish peroxidase (HRP) was then used to detect phosphorylated signals.

Confocal immunofluorescence staining

Pretreated cells were harvested, processed, and stained for confocal immunofluorescence microscopy as previously reported (Gao et al. 2012). Briefly, fixed cells were incubated with Phospho-p44/42 MAPK antibody (1:100, #9101, Cell Signaling) at 4°C overnight, followed by incubation with Alexa Fluor® 594 goat anti-rabbit IgG (red fluorescence) secondary antibody (1:3000, A11037, Molecular Probes) at room temperature for 1 h. After counterstaining with DAPI (D1306, Molecular Probes) for 30 min, slides were examined under a Zeiss LSM510-UV meta (Carl Zeiss).

Statistical analysis

All experiments were performed at least three times in duplicate. Results are expressed as mean±SEM. Cell proliferation data were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test. Luciferase assays data and MTS data with the presence or absence of PD were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test. Two-tailed Student's t-tests were used to compare pairs of time points for data on phosphorylation and RTKs expression (SAS 9.3, SAS Institute). For binding assay, concentrations producing 50% of

the maximum inhibition (IC_{50}) were estimated from Hill models using Prism® 6.02 (GraphPad Software). P values less than 0.05 were considered statistically significant.

Results

Effects of Cd on cell proliferation in ht-UtLM cells and ht-UtSMCs

To evaluate the effects of Cd exposure on proliferation of human ht-UtLM cells and ht-UtSMCs, we conducted MTS proliferation assays with Cd at concentrations of 0.0001 μM to 200 μM. Compared to vehicle controls, ht-UtSMCs and ht-UtLM cells showed statistically significant proliferative responses as measured by absorbance at 490 nm, after incubating with Cd for 24 h, 48 h and 72 h (Figures 1A and 1B). Therefore, we chose two representative intermediate doses (0.1 and 10 μM) to carry out further mechanistic studies.

Cd is less likely to directly influence either ER α or β responses *in vitro*

Next, we sought to test whether ERs are involved in the proliferative effects observed in ht-UtLM cells and ht-UtSMCs following Cd exposure. First, we conducted competitive binding assays to examine the binding affinity of Cd to ERα and ERβ. Cadmium's affinity to bind ERα or ERβ was non-detectable at concentrations ranging from 0.01 nM to 10 mM, while E_2 bound to ERα and ERβ with high affinity (calculated IC_{50} of about 1.04 nM and 0.93 nM, respectively) (See Supplemental Material, Figures S1A and S1B). Next, we determined whether Cd could modulate ER-dependent gene regulation in ht-UtLM cells and ht-UtSMCs. By using a luciferase reporter system, we found that 10 nM E_2 resulted in significant responses in ERE-mediated luciferase activity in hERα and hERβ, which was fully abrogated by 1.0 μM ICI; however, Cd had a negligible influence on hERα or hERβ luciferase activity in ht-UtLM cells or ht-UtSMCs (See Supplemental Material, Figures S2A, S2B, S2C, S2D). Collectively, these results do not

support that Cd directly interacts with either ERα/β *in vitro*. Therefore, we speculate that non-classical ER mechanisms might be responsible for the proliferative effects observed in ht-UtLM cells and ht-UtSMCs induced by Cd.

p44/42 MAPK pathway is essential for Cd-induced cell proliferation in ht-UtSMCs and ht-UtLM cells

The MAPK pathway has been well recognized as a critical mediator of cell proliferation in both normal growth and tumorigenic overgrowth, and reported to be activated after exposure to Cd (Ali et al. 2010). Therefore, we evaluated the influence of Cd on activation of p44/42 MAPK in ht-UtLM cells and ht-UtSMCs. By using western blotting, we found that 0.1 μM and 10 μM Cd resulted in marked increases in phosphorylation of p44/42 MAPK as early as 10 minutes in ht-UtLM cells ($p<0.01$, vs. 0 min) (Figures 2A and 2B) and in ht-UtSMCs ($p<0.01$, vs. 0 min) as well (Figures 2C and 2D). These data show that p44/42 MAPK pathway is undoubtedly activated by Cd and occurs as an early event in both ht-UtLM cells and ht-UtSMCs.

Next, we evaluated whether the activation of the p44/42 MAPK pathway plays a role in Cd-induced cell proliferation. By adding a specific ERK inhibitor (10 μM of PD) prior to Cd treatment (0.1 and 10 μM), Cd-induced cell proliferation was substantially abolished ($p<0.05$, vs. Cd alone) in both cells types (Figures 3A and 3B). As shown in Figure 3C, Cd at 10 μM resulted in robust activation of p44/42 MAPK as indicated by intense red positive-signals in ht-UtLM cells and ht-UtSMCs (Figures 3C-b and 3C-f), while PD dramatically inhibited phospho-p44/42 MAPK expression (Figures 3C-c and 3C-g). Cd administration in the presence of PD did not result in activation of p44/42 MAPK (Figures 3C-d and 3C-h). Taken together, these data suggest that Cd-induced cell proliferation in ht-UtLM cells and ht-UtSMCs is mediated by activation of p44/42 MAPK.

Cd-induced p44/42 MAPK phosphorylation is EGFR-dependent in ht-UtLM cells

It has been well known that various cell surface growth factor receptors, receptor tyrosine kinases (RTKs) can trigger the p44/42 MAPK cascade and phosphorylation. In an attempt to identify specific upstream RTKs involved in Cd-induced p44/42 MAPK activation, we used phosphorylation RTK arrays on ht-UtLM cells and ht-UtSMCs incubated with 10 μM of Cd for 10 min. Among 42 RTKs, 7 candidate proteins were significantly expressed ($p<0.05$, vs. 0 min) in ht-UtLM cells, with epidermal growth factor receptor (EGFR) most highly expressed at baseline, and phosphorylation significantly increased at 10 min after Cd exposure (Figure 4A). The phosphorylated RTKs were differentially expressed in ht-UtSMCs compared to ht-UtLM cells following Cd treatment. In ht-UtSMCs, the most highly phosphorylated RTKs were Ephrin receptors, which maintain a critical role in angiogenesis (see Supplemental Material, Figure S3).

Due to recent studies whereby EGFR has been reported to mediate Cd-induced cell proliferation and survival (Carpenter and Jiang 2013; Martinez Flores et al. 2013), we sought to further determine the contribution of EGFR phosphorylation in p44/42 MAPK activation in the ht-UtLM cells. Cd treatment resulted in phosphorylation of EGFR, which was largely disrupted by the addition of AG1478 (AG; 1 μM), a selective EGFR-RTK inhibitor (Figures 4B and 4C). Accordingly, p44/42 MAPK activation induced by Cd was substantially abolished in the presence of AG1478 as well (Figures 4D and 4E). In short, these data suggest that Cd-induced p44/42 MAPK activation is EGFR-dependent in ht-UtLM cells.

Discussion

Cd is a heavy metal toxin, associated with ubiquitous air and water pollution, as well as a contaminant of cigarette smoke. Circulating levels of Cd in chronically exposed women are

reported to be as high as 0.33-3.5 μg/L (Nasiadek et al. 2011). Moreover, tissue concentrations of Cd are recorded at even higher levels (0.047 and 0.075 μg Cd/g wet tissue in leiomyoma and myometrium, respectively) than those concentrations reported for blood Cd (Pollack et al. 2011). In this study, we found that environmentally relevant concentrations of Cd sufficiently induced cell proliferation in estrogen-responsive ht-UtLM cells and ht-UtSMCs. These effects were less likely mediated through direct interactions with ERα and ERβ, but rather by activation of the p44/42 MAPK pathway. These data suggest that Cd should be considered as an environmental risk factor for uterine fibroids, and that EGFR could be a potential target in managing this risk. The current findings add benign tumors such as uterine fibroids to a long list of targets and adverse effects of Cd exposure.

The acute toxic effects of high concentrations of heavy metals such as lead and arsenic have long been acknowledged as life threatening. Moreover, considerable efforts have been invested in exploring the adverse health effects of low-level and chronic exposures to heavy metals, recently. For example, it has been shown that long-term, low-level lead exposures in children leads to compromised neurobehavioral-cognitive capabilities (Olympio et al. 2009), and that chronic Cd exposure is associated with cancerous transformation of epithelial cells *in vitro* (Benbrahim-Tallaa et al. 2009; Jing et al. 2012). However, attempts to demonstrate the endocrine disrupting or estrogenic effects of low-level Cd exposure in *in vitro* studies have produced inconsistent results (Höfer et al. 2010; Isidori et al. 2010; Silva et al. 2006). Nevertheless, these complexities coincidently support the notion that further investigations regarding the effects of Cd on human health, including the endocrine and reproductive systems hold significant interest and urgency.

Regarding female reproductive health, we have found that Cd can induce cell proliferation in ht-UtLM cells and ht-UtSMCs in a classical ER-independent manner. Previous studies have

reported that Cd has estrogen-like activity and acts as an endocrine disrupting chemical (EDC) (Höfer et al. 2010; Kluxen et al. 2012). Environmentally relevant doses of Cd have been found to induce several estrogenic responses both in cultured breast cancer cell lines and in rats via the ER (Höfer et al. 2010; Kluxen et al. 2012; Siewit et al. 2010; Zang et al. 2009). In contrast, our data consistently showed that Cd does not directly bind to human ERα or ERβ, and has no significant ER transcriptional activity in the presence or absence of ICI. These negative, but important findings suggest that the effects of Cd on ht-UtLM cells and ht-UtSMCs most likely occur in a non-classical ER manner, without significant contributions from ER binding or transactivation. Our findings are in agreement with several groups, in that we and others (Ali et al. 2010; Ali et al. 2012) have found that the estrogenic effects of Cd may be mediated, in part, by the MAPK/ERK1/2 signaling pathway. Ali et al. (2010 and 2012) ruled out classical ER signaling through ERE-regulated genes in Cd-induced estrogenic responses observed *in vivo* and found activation of MAPK pathways as a mode of action for Cd. In human breast cancer cell lines, rapid activation of ERK1/2 and AKT was thought to occur through membrane ERα and GPR30, suggesting there is crosstalk between hormone and growth factor signaling pathways involved in Cd-induced cell signaling (Liu et al. 2008).

There are a number of factors that might account for the above differences in observations regarding Cd's estrogenicity. Variability and lack of standardized protocols for ER binding and transactivation assays make inter-laboratory comparisons and validations difficult (Silva et al. 2006). Also, variations in ER content, transcription factors, and coregulators present in diverse cell types utilized in *in vitro* studies may explain the differences in estrogenic responses observed with Cd treatment (Heldring et al. 2007; Wilson et al. 2004).

Another important finding in our study is that EGFR-dependent p44/42 MAPK activation appears to be critical in Cd-induced cell proliferation in ht-UtLM cells. MAPK pathways are evolutionarily conserved kinase modules that link extracellular signals to the machinery that controls fundamental cellular processes such as normal growth, proliferation, and differentiation (McKay and Morrison 2007). The ERK1/2 pathway is the best studied of the mammalian MAPK pathways, and is dysregulated in approximately, one-third of all human tumors including uterine fibroids. Activation of RTK/MAPK pathways has been well documented in the development of uterine fibroids in our group (Di et al. 2008; Yu et al. 2008) as well as by other investigators (Jiang et al. 2010). Interestingly, we found that EGFR-phosphorylation was upregulated by Cd, which mimics the effects of E_2 in that it has been previously reported that E_2 can upregulate EGFR expression in cultured human uterine leiomyoma cells and we have found similar results in fibroid tissue samples from women in the proliferative (estrogenic) phase of the menstrual cycle (Shimomura et al. 1998). Other investigators have also shown an association between Cd exposure and induction MAPK (Ali et al. 2010) and EGFR expression (Kundu et al. 2011).

Given the fact that EGFR is a critical molecule linked with multiple human tumors, our findings may have many important clinical implications (Ciarmela et al. 2011). It is possible that Cd may have synergistic effects on uterine fibroids in the setting of combinatorial exposures to other EGFR-inducers/activators, such as estrogen. Additionally, it is promising that interventions targeting EGFR might be a meaningful measure in managing the risk of Cd exposure in the setting of uterine fibroids and other disorders (Ciardiello and Tortora 2008; Paez et al. 2004). Besides these implications, there are also several other important directions that should be encouraged in this field. As excessive extracellular matrix (ECM) is another critical feature of uterine fibroids, it may be extremely helpful to evaluate the full spectrum of risks of Cd on

fibroids by exploring the potential effects of Cd on ECM turnover in ht-UtLM cells and ht-UtSMCs. Also, optimized animal studies and human cohort studies may strengthen the notion that Cd is an environmental estrogen mimic and a risk factor for uterine fibroids and other reproductive tract diseases.

In summary, we report that Cd is a potential risk factor for uterine fibroids by potentiating cell proliferation in ht-UtLM cells, via an EGFR-dependent ERK activation pathway, but not through direct interactions with ERα or ERβ.

Conclusions

The present study shows that Cd-induced growth in ht-UtLM cells and ht-UtSMCs was not mediated by a classical ER mechanism of receptor binding and ERE-mediated gene activation, but through nongenomic pathways involving differential activation of growth factor receptors and subsequent MAPK/ERK1/2 phosphorylation (Figure 5). Our results suggest that Cd is a potential environmental risk factor for uterine fibroids. Further exploration of Cd-induced nongenomic signaling and the interaction between the different signaling pathways may be critical for developing new preventive strategies and risk assessment exposure paradigms for fibroids and other hormonally regulated disorders and cancers.

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Figure Legends

Figure 1. The effects of Cd on cell growth in ht-UtLM cells (A) and ht-UtSMCs (B). Cd concentrations less than 50 μM significantly increased cell growth whereas Cd levels equal to or greater than 50 μM significantly inhibited cell growth at 24 h, 48 h, and 72 h in both cell lines. $^{a}p<0.05$, vs. Con at 24 h; $^{b}p<0.05$, vs. Con at 48 h; $^{c}p<0.05$, vs. Con at 72 h. The experiments were repeated three times with independent cultures. Data were collected with an absorbance wavelength of 490 nm.

Figure 2. Cd activates the phosphorylation of p44/42 MAPK (pMAPK). Ht-UtLM cells (A, B) treated with 0.1 or 10 μM Cd showed increased phosphorylation of p44/42 MAPK at 10 min and 60 min. In ht-UtSMCs (C, D), Cd stimulated phosphorylation of p44/42 MAPK at 10 min and 60 min. *p<0.05, vs. 0 min, All the data were repeated at least three times with independent cell cultures.

Figure 3. PD98059 (PD) abrogates Cd-induced cell proliferation and diminishes phosphorylation of p44/42 MAPK. Cell proliferation was evaluated in ht-UtLM cells (A) and ht-UtSMCs (B) treated with vehicle control (Con), 0.1 μM or10 μM Cd and 10 μM PD98059 (PD) alone, or Cd (0.1 μM or10 μM) in combination with 10 μM PD for 72 h. The experiments were repeated three times with independent cultures. Absorbance values were determined at a 490 nm wavelength. Results are presented as mean+SEM (n = 6). *p<0.05, vs. Con. Confocal images (C) of ht-UtLM cells (a, b, c, d) and ht-UtSMCs (e, f, g, h) treated with vehicle control (Con; a and e), 10 μM Cd (b and f), PD (50 μM, c and g), and Cd with PD (d and h) for 10 min. Red: Phospho-p44/42 MAPK, Blue: DAPI; bar = 50 μm.

Figure 4. Cd induces the phosphorylation (p) of Receptor Tyrosine Kinases (RTKs), and EGFR (pEGFR) and p44/42 MAPK (pMAPK) activation in ht-UtLM cells. A. Expression of growth factor RTKs in ht-UtLM cells. Growth factor RTKs were highly expressed after Cd (10 μM) treatment for 10 min in ht-UtLM cells. The upregulated RTKs were EGF Receptor (EGFR), HGF Receptor (HGFR), VEGF Receptor (VEGFR1), FGF Receptor (FGFR2α), ROR (ROR1), PDGF Receptor beta (PDGFRβ), and Insulin Receptor (IR). The Ephrin (Eph) receptors, EphA2 and EphA1 were not significantly expressed in ht-UtLM cells following Cd exposure. The array was repeated at least 3 times. The bars represent the dot blot intensity values for ht-UtLM cells.

*p<0.05, vs. 0 min. Ht-UtLM cells treated with 0.1 or 10 μM Cd showed increased phosphorylation of EGFR (B, C) and phosphorylation of p44/42 MAPK (D, E) at 10 min. In the presence of AG (1 μM), an inhibitor of EGFR, the increased EGFR phosphorylation induced by Cd was abolished. Meanwhile, AG also abrogated Cd-induced phosphorylation of p44/42 MAPK. *p<0.05, vs. 0 min. The experiments were repeated three times with independent cultures.

Figure 5. Schematic diagram of proposed molecular mechanism of EGFR and p44/42 MAPK (MAPK) phosphorylation in Cd-induced cell growth in fibroid cells. Classical estrogen receptor (ER) pathways are not directly involved in Cd-induced cell proliferation in ht-UtLM cells. We have shown that EGFR and MAPK play a role in Cd-induced proliferation in ht-UtLM cells. We propose that Cd mediates the phosphorylation of growth factor Receptor Tyrosine Kinases (RTKs), such as EGFR, HGFR and VEGFR1, which in turn activate downstream effector MAPK. The EGFR inhibitor, AG1478, can diminish Cd-induced EGFR and MAPK phosphorylation. The MAPK inhibitor, PD98059, can also decrease Cd-induced MAPK phosphorylation and cell growth.

Figure 1

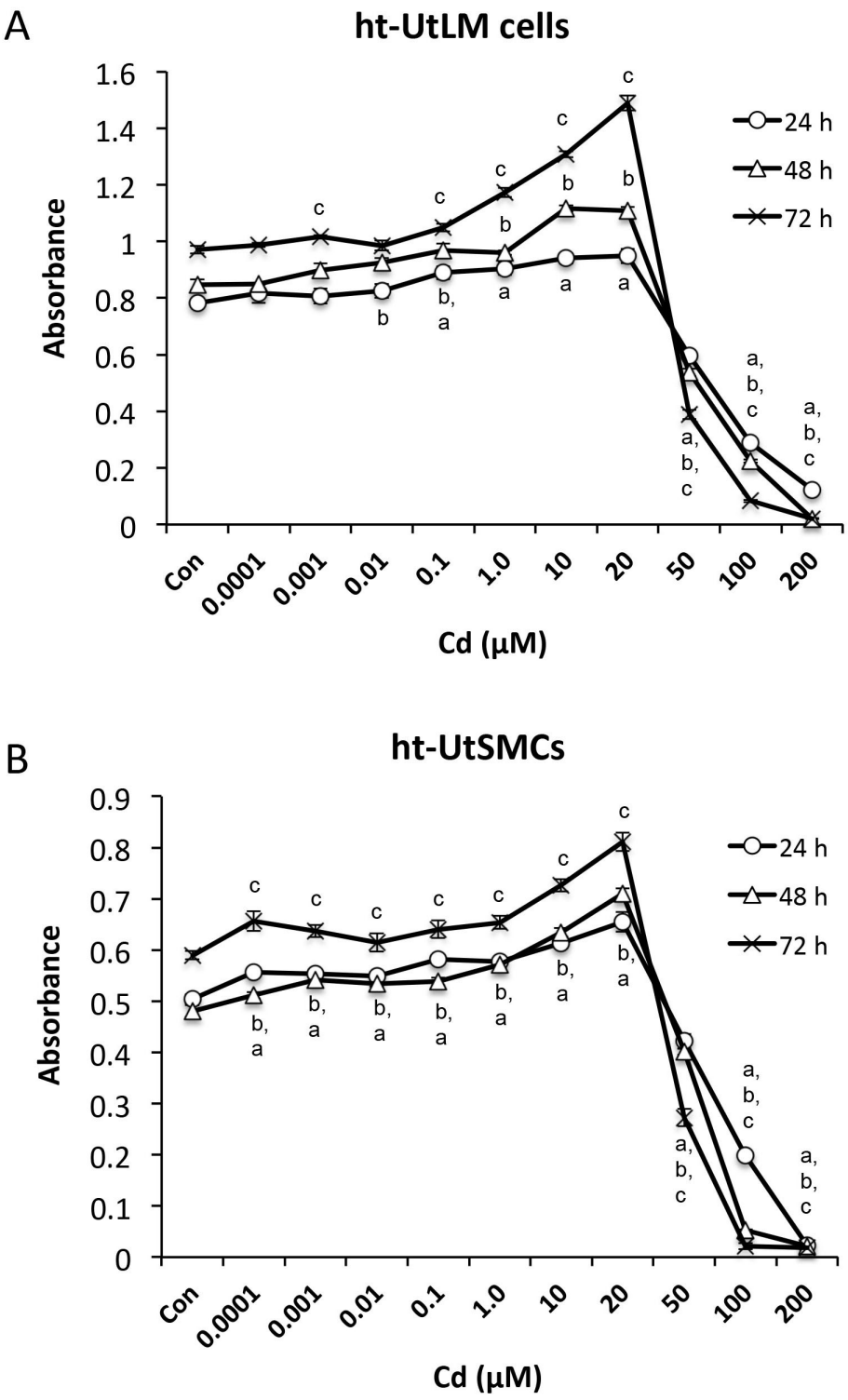

A
ht-UtLM cells
Absorbance
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
24 h
48 h
72 h
Con
0.0001
0.001
0.01
0.1
1.0
10
20
50
100
200
Cd (μM)
B
ht-UtSMCs
Absorbance
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
24 h
48 h
72 h
Con
0.0001
0.001
0.01
0.1
1.0
10
20
50
100
200
Cd (μM)

Figure 2

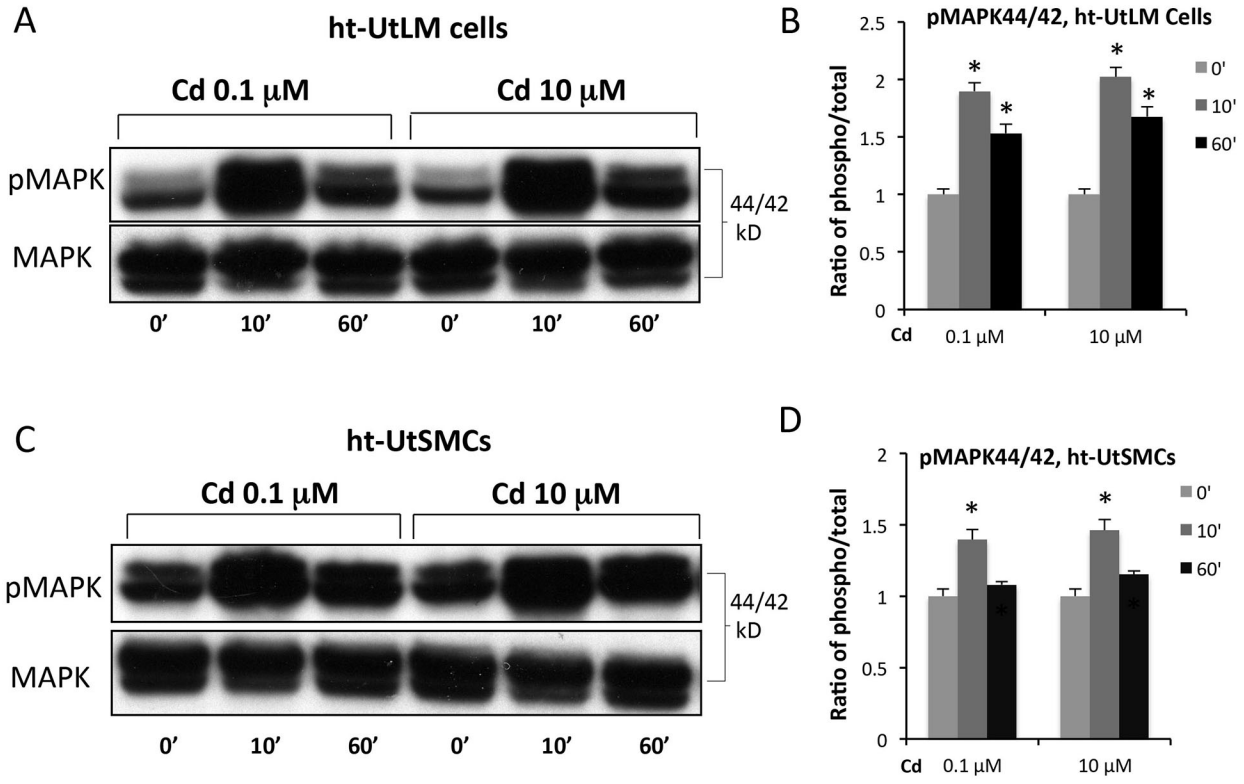

A
ht-UtLM cells
Cd 0.1 μM
Cd 10 μM
pMAPK
MAPK
44/42 kD
0’ 10’ 60’ 0’ 10’ 60’
B
pMAPK44/42, ht-UtLM Cells
Ratio of phospho/total
0'
10'
60'
Cd 0.1 μM 10 μM
C
ht-UtSMCs
Cd 0.1 μM
Cd 10 μM
pMAPK
MAPK
44/42 kD
0’ 10’ 60’ 0’ 10’ 60’
D
pMAPK44/42, ht-UtSMCs
Ratio of phospho/total
0'
10'
60'
Cd 0.1 μM 10 μM

Figure 3

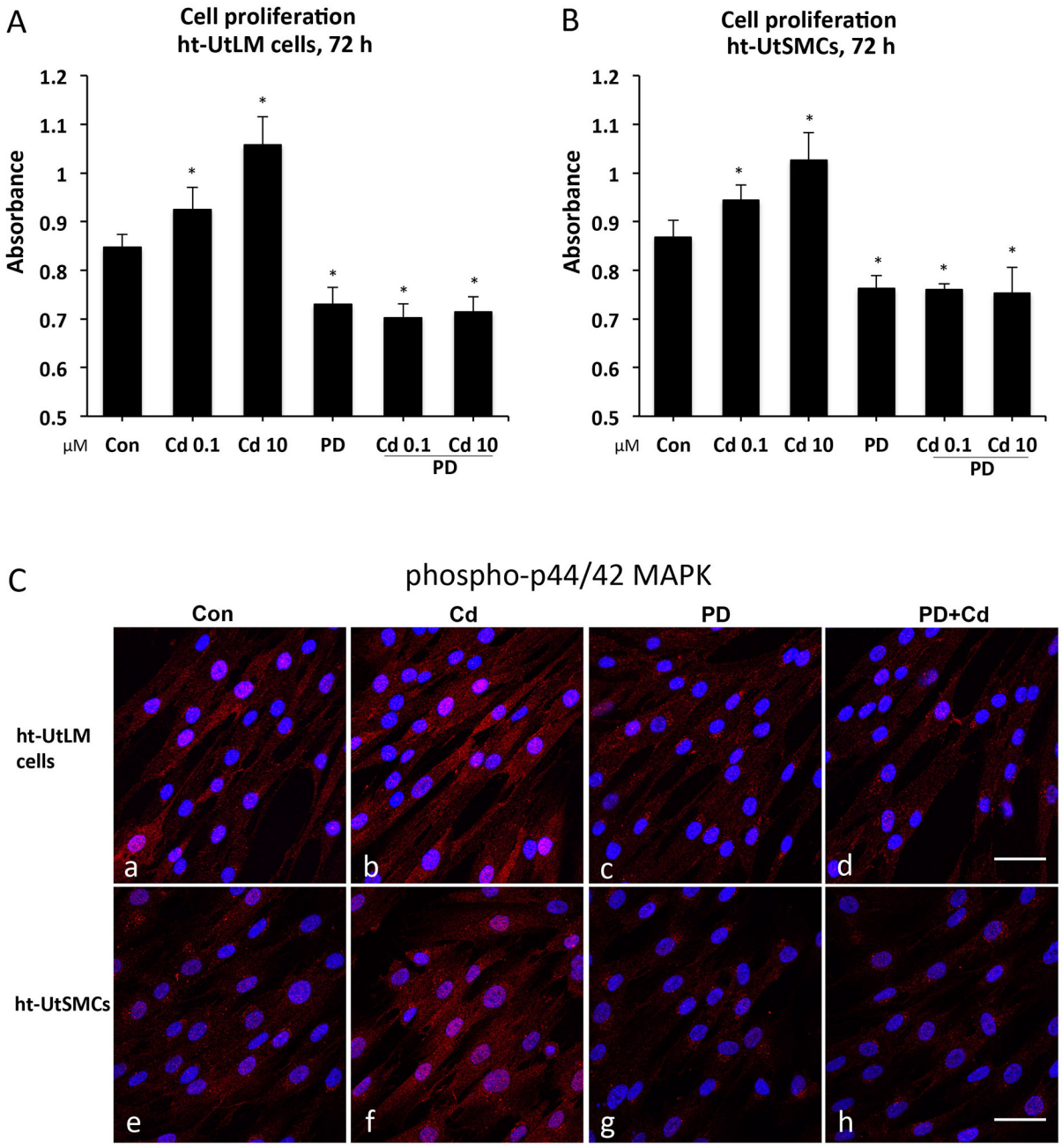

A
Cell proliferation
ht-UtLM cells, 72 h
Absorbance
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
μM Con Cd 0.1 Cd 10 PD Cd 0.1 Cd 10
PD
B
Cell proliferation
ht-UtSMCs, 72 h
Absorbance
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
μM Con Cd 0.1 Cd 10 PD Cd 0.1 Cd 10
PD
C
phospho-p44/42 MAPK
Con
Cd
PD
PD+Cd
ht-UtLM cells
ht-UtSMCs
a
b
c
d
e
f
g
h

Figure 4

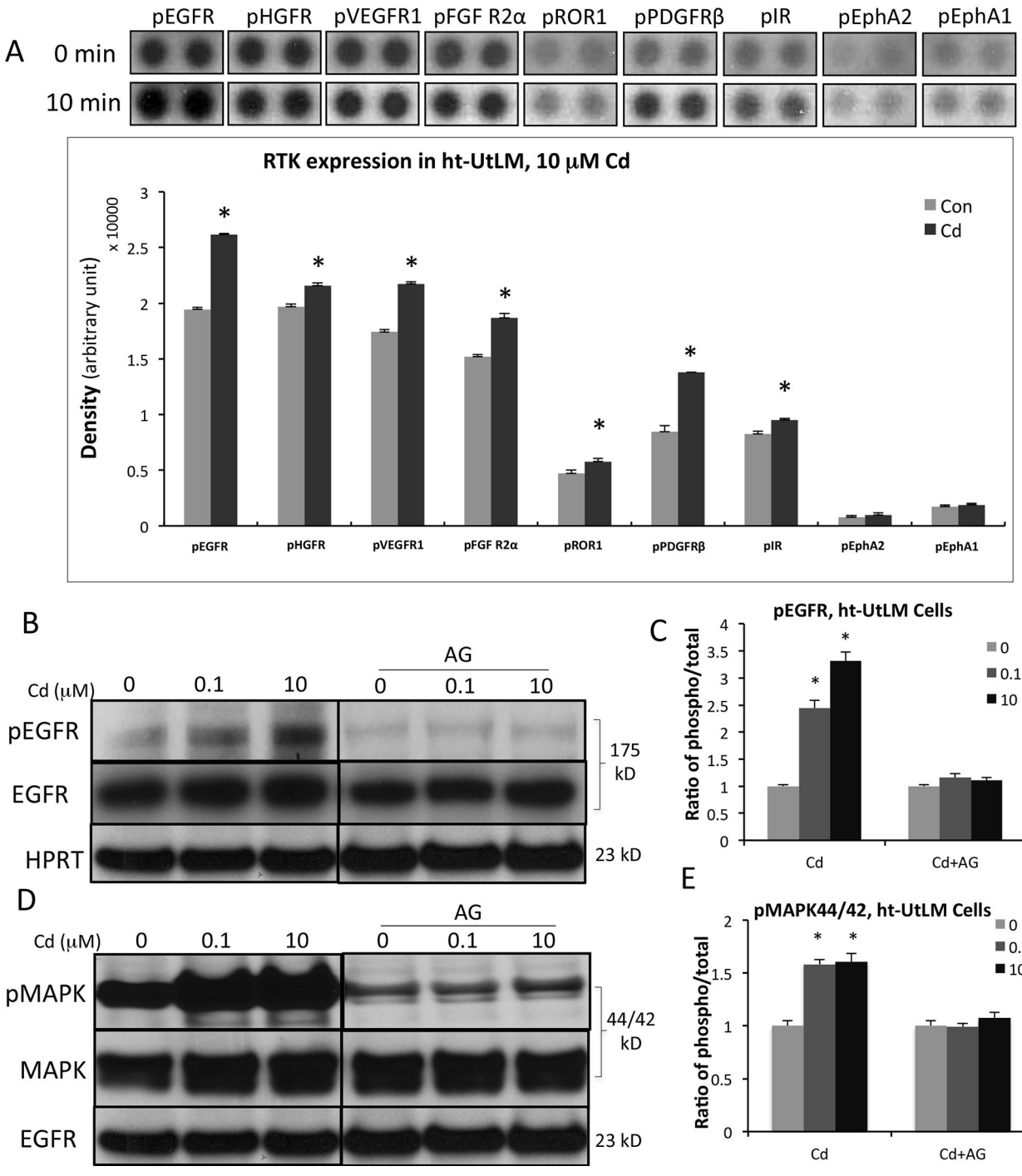
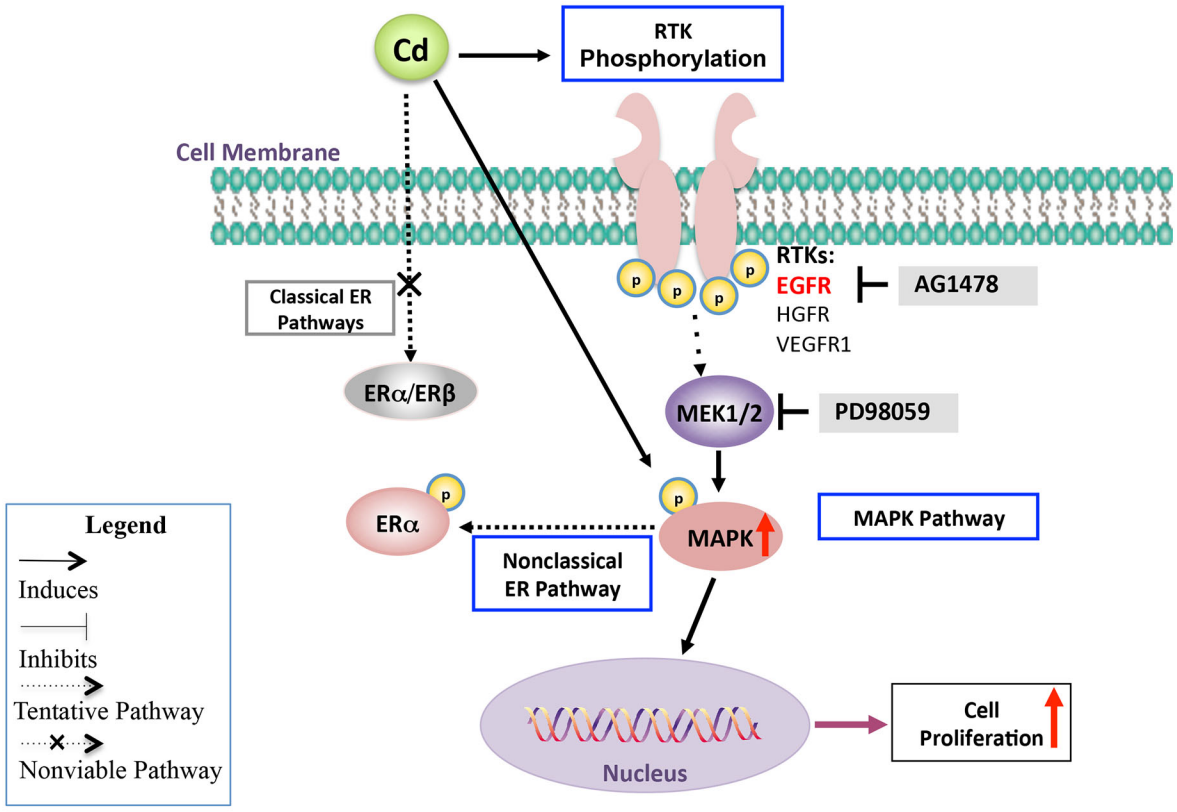

A
0 min
10 min
pEGFR
pHGFR
pVEGFR1
pFGF R2α
pROR1
pPDGFRβ
pIR
pEphA2
pEphA1
RTK expression in ht-UtLM, 10 μM Cd
Density (arbitrary unit)
x 10000
Con
Cd
B
Cd (μM)
0
0.1
10
AG
pEGFR
EGFR
HPRT
175 kD
23 kD
C
pEGFR, ht-UtLM Cells
Ratio of phospho/total
Cd
Cd+AG
D
pMAPK
MAPK
EGFR
44/42 kD
E
pMAPK44/42, ht-UtLM Cells

Figure 5


Cd
RTK
Phosphorylation
Cell Membrane
Classical ER
Pathways
ERα/ERβ
RTKs:
EGFR
HGFR
VEGFR1
AG1478
MEK1/2
PD98059
ERα
MAPK
MAPK Pathway
Nonclassical
ER Pathway
Nucleus
Cell
Proliferation
Legend
Induces
Inhibits
Tentative Pathway
Nonviable Pathway